



Rec'd PCT/PTC 16 MAR 2005  
PCT/AU03/01212

REC'D 30 SEP 2003

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**A U S T R A L I A**

**Patents Act 1990**

**PROVISIONAL SPECIFICATION**

for the invention entitled:

**“A method of treating an autoimmune disease”**

The invention is described in the following statement:

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## **A METHOD OF TREATING AN AUTOIMMUNE DISEASE**

### **FIELD OF THE INVENTION**

5 The present invention relates generally to a method for treating or ameliorating the symptoms of or reducing or otherwise minimizing the risk of development of an autoimmune disease such as but not limited to autoimmune diabetes. More particularly, the present invention relates to the use of genetically modified hemopoietic stem cells and/or hemopoietic progenitor cells which express genetic material encoding one or more  
10 autoantigens which give rise to antigen-presenting cells that induce immune tolerance and/or protective immunity. The present invention provides, therefore, a method for the treatment and/or prophylaxis of autoimmune disease conditions such as type 1 diabetes.

### **BACKGROUND OF THE INVENTION**

15

Bibliographic details of references provided in the subject specification are listed at the end of the specification.

Reference to any prior art in this specification is not, and should not be taken as, an  
20 acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

Insulin-dependent or type 1 diabetes is caused by a lack of insulin, due to autoimmune-mediated destruction of pancreatic islet  $\beta$  cells. Individuals with type 1 diabetes require  
25 regular insulin injections to control their blood glucose levels. Failure to treat individuals in this manner can lead to death.

A more long term treatment strategy is required with the prevention of the autoimmune condition being the principal goal.

30

Pancreas transplantation is currently the only curative therapy for type 1 diabetes, but this

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is hampered by the requirement for potentially toxic, life-long immunosuppressive drugs and by the dearth of human donors.

Bone marrow (BM) or hematopoietic stem cell (HSC) transplantation has recently been  
5 used to treat clinically severe autoimmune disease (Burt *et al.*, *Blood* 99: 768-784, 2002).  
In pre-clinical animal models, effective treatment of spontaneous autoimmune disease  
requires transplantation of BM or HSC from disease-resistant strains. To date, this has  
been achieved by allogeneic BM transplantation (BMT) (Ikehara *et al.*, *Proc. Natl. Acad.*  
*Sci. USA* 82: 7743-7747, 1985; LaFace and Peck, *Diabetes* 38: 894-901, 1989; El-Badri *et*  
10 *al.*, *Transplantation* 70: 870-877, 2000; Himeno and Good, *Proc. Natl. Acad. Sci. USA* 85:  
2235-2239, 1998; Kirzner *et al.*, *Biol. Blood Marrow Transplant.* 6: 513-522, 2000)  
leading to full or mixed chimerism (Li *et al.*, *J. Immunol.* 156: 380-388, 1996; Kaufman *et*  
*al.*, *J. Immunol.* 158: 2435-2442, 1997). However, the requirement for cytotoxic  
conditioning of the host and the risk of graft rejection (Castro-Malaspina *et al.*, *Blood* 99:  
15 1943-1951, 2002) or graft-versus-host disease (Ratanatharathorn *et al.*, *Bone Marrow*  
*Transplant* 28: 121-129, 2001) render this approach unsuitable for widespread clinical  
application.

There is a need, therefore, to develop alternative strategies and approaches which prevent  
20 the development of autoimmune diabetes as well as other autoimmune disease conditions.

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## SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the  
5 inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Targeting antigen to resting antigen-presenting cells (APCs), such as B cells, dendritic cells, epithelial cells or macrophages amongst others, is proposed as a means of inducing  
10 immunological unresponsiveness (tolerance) and/or protective immunity. As all immune cells are derived from hemopoietic stem cells (HSCs) and hemopoietic progenitor cells (HPCs), it is proposed, in accordance with the present invention, that HSCs and/or HPCs encoding an autoantigen will develop into APCs expressing the autoantigen. These are then used as an antigen-specific therapy to prevent autoimmune disease. Transplantation of  
15 syngeneic HSCs and/or HPCs avoids the need for conditioning regimens in a host and represents a novel, safe and effective strategy for preventing or treating autoimmune disease conditions.

With respect to autoimmune diabetes, proinsulin is proposed to be the key autoantigen.  
20

In accordance with the present invention, therefore, syngeneic transplantation of HSCs and/or HPCs encoding proinsulin enables proinsulin expression in resting APCs and this results in the prevention of the development of autoimmune diabetes. This is a safe and effective antigen-specific strategy applicable to autoimmune diabetes as well as other  
25 autoimmune conditions.

Accordingly, the present invention contemplates a method for preventing or otherwise reducing the risk of development and/or reducing the severity of an autoimmune-mediated condition in an animal or avian species. The method involves collecting HSCs and/or  
30 HPCs either from the animal or avian species to be treated or from a compatible donor, genetically modifying some or all of the HSCs and/or HPCs such that they express genetic

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disease and introducing these into the animal or avian species to be treated. Presentation of the autoantigen by APCs is proposed to induce T cell unresponsiveness or tolerance and/or protective immunity. The HSCs and/or HPCs may be collected from bone marrow or isolated from peripheral blood, cord blood or other convenient sites such as the liver. Once  
5 genetically modified, the cells are generally infused into the subject such that they enter the peripheral blood. This route of administration includes infusion or introduction to the liver such as *via* the portal vein.

In a preferred embodiment, the autoimmune disease or condition is type 1 diabetes. The  
10 present invention extends, however, to a range of autoimmune diseases. With respect to autoimmune diabetes, the preferred autoantigen is proinsulin or an antigenic fragment or portion thereof.

The most preferred animal is a human but the present invention extends to other primates  
15 as well as livestock animals, laboratory test animals, companion animals, captured wild animals and avian species such as caged (aviary) birds, poultry birds and game birds.

The present invention provides pharmaceutical kits to facilitate the collection and/or  
20 generation of HSCs and/or HPCs which are then genetically modified to express autoantigens. The pharmaceutical kits preferably include instructions for clinical and/or laboratory use.

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A list of abbreviations used herein is provided in Table 1.

**TABLE 1**  
*Abbreviations*

5

ABBREVIATION	DESCRIPTION
APC	antigen presenting cell
BM	bone marrow
BMT	bone marrow transplantation
CD	cluster differentiation antigens
DC	dendritic cells
F2.5	FCS (2.5% v/v)
FCS	fetal calf serum
G	GM-CSF
G + T	mixture of GM-CSF and TGF $\beta$ 1
GM-CSF	granulocyte macrophage colony stimulating factor
HPC	hemopoietic progenitor cell
HSC	hemopoietic stem cell
i.v.	intravenous
iDC	immature dendritic cells
IL-10	interleukin 10
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
NOD mice	non-obese diabetic mice
NOD-PI mice	NOD-transgenic proinsulin mouse
OVA	ovalbumin
PBL	peripheral blood leukocytes
PI	proinsulin
s.c.	subcutaneous
SD	standard deviation
T	TGF $\beta$ 1
TGF $\beta$ 1	transforming growth factor $\beta$ 1
T1D	type 1 diabetes

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graphical representation showing that transplantation of NOD-PI BM inhibits diabetes development. (A) Diabetes incidence was significantly reduced in recipients of NOD-PI BM (▼) compared to NOD BM (▲) ( $P=0.003$ ) or untreated NOD mice (■) ( $P=0.001$ ). NOD BM recipients did not differ from untreated controls. Data are pooled from two experiments in which BMT from NOD-PI or NOD mice was performed in parallel. (B) Diabetes incidence was significantly reduced in recipients of T cell-depleted NOD-PI BM (▼) compared to T cell-depleted NOD BM (▲) ( $P=0.003$ ) or untreated NOD mice (■) ( $P=0.036$ ). NOD BM recipients did not differ from untreated controls. Data are pooled from three experiments in which BMT from NOD-PI or NOD mice was performed in parallel.

Figure 2 presents micrographic and graphical representations showing that transplantation of T cell-depleted NOD-PI BM prevents insulinitis but not sialitis. (A) Islets free of inflammatory infiltrate (insulitis) were common in recipients of NOD-PI BM and infiltration, when present, was restricted to the periphery of islets (arrow). (B) Extensively infiltrated islets (\*) were common in recipients of NOD BM. (C) Insulitis was significantly reduced in recipients of T cell-depleted NOD-PI BM (▲) compared to NOD BM (▼) cells ( $P = 0.008$ ). Data for age-matched NOD (■) and NOD-PI (◆) mice are included for comparison. (D) The number of sublingual gland inflammatory foci (sialitis score) did not differ between BMT and untreated mice. Individual mouse scores are pooled from two experiments in which BMT from NOD and NOD-PI mice was performed in parallel (horizontal bar indicates mean).

Figure 3 presents graphical representations showing that transfer of NOD-PI hematopoietic stem cells (HSC) or hematopoietic progenitor cells (HPC) prevents diabetes development. (A) diabetes incidence was significantly reduced in recipients of NOD-PI HSC (▼) compared to recipients of NOD HSC (▲) ( $P=0.019$ ) or untreated NOD mice (■) ( $P=0.029$ ). NOD HSC recipients and untreated NOD mice did not differ significantly. (B) Diabetes incidence was significantly reduced in recipients of NOD-PI HPC (▼) compared



to recipients of NOD HPC (▲) ( $P=0.035$ ) or untreated NOD mice (■) ( $P=0.021$ ). NOD HPC recipients and untreated NOD mice did not differ significantly. Data are from one (A) or two (B) experiments in which BMT from NOD-PI and NOD mice was performed in parallel.

5

Figure 4 provides a graphical representation and tabulated data showing reconstitution of peripheral blood leucocytes (PBL) in recipients of T cell-depleted NOD or NOD-PI BM. (A) PBL were markedly depleted at 10-14 days but reconstituted by 8 weeks after irradiation and BMT in both NOD (▼) and NOD-PI (▲) T cell-depleted BM recipients, compared to untreated age-matched NOD mice (■). (B) PBL subsets in recipients of T cell-depleted BM from NOD or NOD-PI donors reconstituted similarly and were similar to age-matched NOD controls. Data are mean  $\pm$  SD from two experiments in which BMT from NOD and NOD-PI mice were performed in parallel.

15 Figure 5 is a graphical representation showing T cell recall responses to ovalbumin (OVA) immunization. Mice were immunised subcutaneously with OVA 100 days post-BMT and recall responses of splenic T cells measured 14 days later. T cell proliferation in the presence of OVA was similar for age-matched control NOD mice (■) and recipients of T cell-depleted NOD (▼) or NOD-PI (▲) BM. Open symbols indicate proliferation in the absence of OVA. Data are from individual mice pooled from three separate experiments in which NOD and NOD-PI BMT were performed in parallel.

Figure 6 shows flow cytometric profiles indicating the propagation of "immature" BM DC (iDC) in TGF- $\beta$ 1 and IL-10. As a source of iDC, BM was cultured in GM-CSF and TGF- $\beta$  (G+T). iDC from G+T BM expressed low levels of MHC class II and co-stimulation molecules and were weak stimulators in the mixed lymphocyte reaction (MLR).

Figure 7 is a graphical representation showing that G+T BM from NOD-PI, but not control NOD mice, significantly inhibited ( $p<0.01$ ) diabetes development when transferred i.v. to 4 week-old female NOD mice.

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**Figure 8** is a series of flow cytometric dot blots revealing that G+T BM contained large numbers of undifferentiated CD11c<sup>-</sup>/CD11b<sup>+</sup>/Gr-1<sup>+</sup> myeloid cells, in addition to CD11c<sup>+</sup>/CD11b<sup>+</sup>/Gr-1<sup>-</sup> iDC.

- 5 **Figure 9** is a graphical representation showing proinsulin-encoding Gr-1<sup>+</sup> cells inhibits diabetes treated at four weeks.

**Figure 10** is a graphical representation showing proinsulin-encoding Gr-1<sup>+</sup> cells inhibits diabetes treated at four weeks ( $1.8 \times 10^6$  CD11c-depleted i.v.)

## **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

The present invention provides a safe and effective protocol for treating and/or preventing autoimmune disease conditions.

5

The protocol generally involves the steps of:-

- (i) collecting a sample of HSCs and/or HPCs from an individual;
- 10 (ii) genetically modifying all or some of the HSCs and/or HPCs so that the HSCs and/or HPCs produce one or more autoantigens associated with the autoimmune disease; and
- (iii) introducing the genetically modified HSCs and/or HPCs into the same individual or  
15 a compatible individual and which then eventually become APCs expressing the autoantigen.

The above steps may be combined and/or the order changed. Additional steps may also be included.

20

Reference to an "individual" includes a human individual as well as an animal or avian subject. The terms "individual" and "subject" in relation to the animal being treated may be used interchangeably. An "animal" includes a human, primate, livestock animal (e.g. sheep, horse, cow, horse, donkey, goat, pig), laboratory test animal (e.g. rabbit, mouse, rat,  
25 guinea pig), companion animal (e.g. dog, cat) or captured wild animal. An "avian species" includes caged or aviary birds, poultry birds (e.g. chickens, bantams, geese, turkeys) and game birds.

The most preferred animal in terms of medical science is a human. The present invention  
30 extends, however, to veterinary uses of the protocol to reduce autoimmune disease conditions in non-human animals.

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The HSCs and/or HPCs are generally obtained from a sample of bone marrow such as from drilling into the hip bone. However, the present invention further extends to isolating and where necessary sorting HSCs and HPCs from peripheral blood including cord blood  
5 and blood from the liver. The cells are generally introduced into the recipient *via*, for example, i.v. injection or infusion into the peripheral blood system or liver *via* the portal vein. However, direct introduction into a recipient's bone marrow, although not preferred, is nevertheless contemplated by the present invention.

10 The process of the present invention may be "syngeneic", "allogeneic" or "xenogeneic" with respect to the individuals within an animal species from which HSCs and/or HPCs are isolated and the individuals who receive the cells. A "syngeneic" process means that the individual from which the HSCs and/or HPCs are derived has the same MHC genotype as the recipient of the genetically modified HSCs and/or HPCs. An "allogeneic" process is  
15 where the HSCs and/or HPCs are from a MHC-incompatible individual to the individual to which the HSCs and/or HPCs are to be introduced. A "xenogeneic" process is where the HSCs and/or HPCs are from a different species to that to which the HSCs and/or HPCs are introduced. Preferably, the method of the present invention is conducted as a syngeneic process. To the extent that either an allogeneic or xenogeneic process is utilized, it should  
20 be understood that it may be necessary to modify the protocol such that any immunological responses, which may occur due to the mixing of foreign immuno-competent cells, are minimized.

Accordingly, the present invention contemplates a method for preventing or otherwise  
25 minimizing the risk of development of or reducing the severity of an autoimmune condition in a subject, said method comprising introducing into said subject, HSCs and/or HPCs which have been genetically modified such that they now produce one or more autoantigens associated with the autoimmune condition.

30 The HSCs and/or HPCs are developed into APCs expressing particular autoantigens. Examples of APCs include but are not limited to dendritic cells, B-lymphocytes, epithelial

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cells or macrophages.

As indicated above, the subject includes a human, non-human animal and avian subject. Preferably, the subject is a human. The subject (e.g. human) may have pre-clinical diabetes  
5 or may be at risk of developing diabetes or may have clinical diabetes.

Also as indicated above, the method may involve the syngeneic, allogeneic or xenogeneic administration of HSCs and/or HPCs to a subject. However, a syngeneic protocol is preferred.

10

Accordingly, in a preferred embodiment, the present invention provides a method for preventing or otherwise minimizing the risk of developing or reducing the severity of an autoimmune disease in a subject, said method comprising introducing into said subject syngeneic HSCs and/or HPCs which have been genetically modified to produce one or  
15 more autoantigens associated with the autoimmune condition.

The preferred autoimmune disease is autoimmune diabetes, also known as type 1 diabetes or insulin-dependent diabetes. However, the present invention extends to the use of the subject protocol in the treatment of a range of autoimmune conditions. The only criterion is  
20 that an autoantigen associated with the disease condition be known. Examples of autoimmune conditions contemplated herein include *inter alia* systemic lupus, Crohn's disease, cardiomyopathy, hemolytic anemia, fibromyalgia, Graves' disease, ulcerative colitis, vasculitis, multiple sclerosis, myasthenia gravis, myositis, neutropenia, psoriasis, chronic fatigue syndrome, juvenile arthritis, juvenile diabetes, scleroderma, psoriatic  
25 arthritis, Sjogren's syndrome, rheumatic fever, rheumatoid arthritis, sarcoidosis, idiopathic thrombocytopenic purpura (ITP), Hashimoto's disease, mixed connective tissue disease, interstitial cystitis, pernicious anemia, leukoencephalitis, alopecia areata, ankylosing spondylitis, primary biliary cirrhosis, anti-GBM nephritis, anti-TBM nephritis, anti-phospholipid syndrome, polymyalgia rheumatica, polymyositis, autoimmune  
30 Addison's disease, chronic active hepatitis, vitiligo, autoimmune hyperlipidemia, autoimmune myocarditis, temporal arteritis, autoimmune thyroid disease, axonal and

neuronal neuropathies, Behçet's disease, bullous pemphigoid, allergic asthma, osteoarthritis, Chagas' disease, uveitis, chronic inflammatory demyelinating polyneuropathy (CIDP), cicatricial pemphigoid/benign mucosal pemphigoid, Cogan's syndrome, congenital heart block, Coxsackie myocarditis, demyelinating neuropathies, 5 dermatomyositis, discoid lupus, phacoantigenic uveitis, polyarteritis nodosa, Dressler's syndrome, essential mixed cryoglobulinemia, Evan's syndrome, Goodpasture's syndrome, allergic rhinitis, Guillain-Barré syndrome, hypogammaglobulinemia, inclusion body myositis, vesiculobullous dermatosis, Wegener's granulomatosis, Ménière's disease, Lambert-Eaton syndrome, Mooren's ulcer, non-typical celiac disease, ocular cicatricial 10 pemphigoid, pemphigus vulgaris, perivenous encephalomyelitis, post-pericardiotomy syndrome, scleritis, sperm and testicular autoimmunity, Stiff man's syndrome, subacute bacterial endocarditis (SBE), sympathetic ophthalmia, transverse myelitis and necrotizing myelopathy, type 1 autoimmune polyglandular syndrome, type II autoimmune polyglandular syndrome, pernicious anaemia and endometriosis.

15

According to a preferred embodiment, the present invention contemplates a method of preventing, minimizing the risk of development of or the severity of autoimmune diabetes in a human subject, said method comprising administering to said human subject an effective amount of HSCs and/or HPCs isolated from said human subject or from a 20 syngeneic individual and which HSCs and/or HPCs have been genetically modified such that they express an autoantigen associated with autoimmune diabetes.

The preferred autoantigen is proinsulin or a homolog or antigenic derivative, part, fragment or portion thereof. The proinsulin is generally of human origin although 25 humanized proinsulin molecules from, for example, pigs, sheep, horses, goats, mice or rats are also contemplated.

According to a most preferred embodiment, the present invention provides a method of preventing, minimizing the risk of development of or the severity of autoimmune diabetes 30 in a human subject, said method comprising administering to said human subject an effective amount of HSCs and/or HPCs isolated from said human subject or from a

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syngeneic individual and which HSCs and/or HPCs have been genetically modified such that they produce proinsulin.

5 It is proposed herein that syngeneic transplantation of gene-modified HSC and/or HPCs is a novel approach to antigen-specific immunotherapy which advances the principle of regulating autoimmune disease from within the hematopoietic compartment.

10 Preferably, the autoimmune disease is diabetes and the autoantigen is proinsulin since proinsulin contains T cell epitopes implicated in human (Rudy *et al.*, *Mol. Med.* 1: 625-633, 1995) and mouse (Chen *et al.*, *J. Immunol.* 167: 4926-4935, 2001) type 1 diabetes. In work leading up to the present invention, the inventors observed that NOD mice transgenically- expressing proinsulin targeted to APCs by an MHC class II promoter (French *et al.*, 1997, *supra*) contained bone marrow which could be used to adoptively transfer protection against the development of autoimmune diabetes following bone marrow transplantation to a wild-type NOD mouse. Protection from diabetes was profound in recipients of bone marrow from NOD-PI mice. By transferring highly purified HSC or HPCs, protection can be attributed to the APC progeny of engrafted HSCs/HPCs rather than other potentially immunoregulatory cells transferred in whole or T cell-depleted bone marrow. Most importantly, transfer of small numbers of genetically-modified HSCs totally prevents diabetes. HSC transplantation also demonstrates that diabetes transferred by wild-type NOD HSC is due to generation of diabetogenic T cells *de novo* rather than to transfer of diabetogenic T cells. Nevertheless, diabetogenic T cells clearly either do not develop or fail to acquire effector function in mice destined to express proinsulin in APC. These results provide proof of principle for genetically-modified HSCs as a therapeutic tool for autoimmune disease prevention.

30 The context in which antigen presentation occurs controls the balance of T cell immunity (Garza *et al.*, *J. Exp. Med.* 191: 2021-2027, 2000; Frazer *et al.*, *J. Immunool* 167:6180-6187, 2001). Antigen presented by resting APC induces inactivation of T cells (Niimi *et al.*, 1998, *supra*; Finkelman *et al.*, 1996; *supra*; Hawiger *et al.*, 2001, *supra*) and inhibits antigen-specific Ab production (Finkelman *et al.*, 1996, *supra*). By transgenically targeting

antigen expression, dendritic cells (DC) have been shown to play a key role in thymic deletion of antigen-specific T cells (Brocker *et al.*, *J. Exp. Med.* 185: 541-550, 1997). Importantly, suppression of T cell responses in the periphery has also been described following administration of DC-targeted antigen (Finkelman *et al.*, 1996, *supra*; Hawiger  
5 *et al.*, 2001, *supra*). The ability to harness peripheral mechanisms of immune tolerance is likely to be the key to autoantigen-specific immunotherapy in individuals with autoreactive T cells.

While the inventors used myeloablative conditioning with irradiation to favour maximum  
10 engraftment of donor HSCs, this would not be acceptable in asymptomatic humans with pre-clinical autoimmune diabetes. However, as no MHC barrier exists, the approach is adaptable to protocols which require no toxic pre-bone marrow transplantation conditioning. By using HSCs derived from transgenic mice, the need to genetically-engineer HSCs *ex vivo*, which has been a major hurdle for HSC therapy, is by-passed. For  
15 human application, vectors capable of effectively transducing HSCs for long-term gene expression after engraftment are required. Gene expression can be effectively targeted to MHC class II<sup>+</sup> APC *in vivo* by lentiviral-vector transduction of human HSC (Cui *et al.*, *Blood* 99: 399-408, 2002). Therefore, a strategy whereby HSCs and/or HPCs are harvested from peripheral blood, optionally following cytokine-induced mobilization, genetically  
20 modified and reinfused is the preferred approach to the therapy of autoimmune disease.

Accordingly, another aspect of the present invention contemplates a method for treating or reducing the risk of development of or reducing the severity of diabetes in a human, said method comprising:-

25

(i) isolating HSCs and/or HPCs from peripheral blood or bone marrow, optionally including the steps of cytokine-mediated mobilization of the HSCs and/or HPCs;

30

(ii) genetically modifying the HSCs and/or HPCs so that the cells now produce proinsulin or an antigenic part, fragment or derivative thereof and continue to do so as APCs; and



- (iii) infusing or introducing the genetically modified cells into a human subject.

- Reference to genetically modifying HSCs and/or HPCs includes introducing nucleic acid molecules encoding proinsulin or other autoantigens into the genome of the cells. Generally, the nucleic acid molecule is DNA. The DNA may encode a full length autoantigen, multiple full length autoantigens or one or more fragments of one or more autoantigens which carry antigenic epitopes.
- 10 Yet another aspect of the present invention provides a vector useful for introducing genetic material encoding an autoantigen such as proinsulin, said vector comprising a nucleotide sequence encoding the autoantigen or an antigenic fragment thereof and a selectable marker.
- 15 A selectable marker in the vector allows for selection of targeted cells that have stably incorporated the autoantigen-encoding DNA. This is especially useful when employing relatively low efficiency transformation techniques such as electroporation, calcium phosphate precipitation and liposome fusion where typically fewer than 1 in 1000 cells will have stably incorporated the exogenous DNA. Using high efficiency methods, such as viral
- 20 vectors and microinjection into nuclei, typically from 5-25% of the cells will have incorporated the DNA; and it is, therefore, feasible to screen the targeted cells directly without the necessity of first selecting for stable integration of a selectable marker. Either isogenic or non-isogenic DNA may be employed.
- 25 Examples of selectable markers include genes conferring resistance to compounds such as antibiotics, genes conferring the ability to grow on selected substrates, genes encoding proteins that produce detectable signals such as luminescence. A wide variety of such markers are known and available, including, for example, antibiotic resistance genes such as the neomycin resistance gene (*neo*) and the hygromycin resistance gene (*hyg*).
- 30 Selectable markers also include genes conferring the ability to grow on certain media substrates such as the *tk* gene (thymidine kinase) or the *hprt* gene (hypoxanthine

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phosphoribosyltransferase) which confer the ability to grow on HAT medium (hypoxanthine, aminopterin and thymidine); and the bacterial *gpt* gene (guanine/xanthine phosphoribosyltransferase) which allows growth on MAX medium (mycophenolic acid, adenine and xanthine). Other selectable markers for use in mammalian cells and plasmids carrying a variety of selectable markers are described in Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbour, New York, USA, 1990.

The selectable marker may depend on its own promoter for expression and the marker gene may be derived from a very different organism than the organism being targeted (e.g. prokaryotic marker genes used in targeting mammalian cells). However, it is preferable to replace the original promoter with transcriptional machinery known to function in the recipient cells. A large number of transcriptional initiation regions are available for such purposes including, for example, metallothionein promoters, thymidine kinase promoters,  $\beta$ -actin promoters, immunoglobulin promoters, SV40 promoters and human cytomegalovirus promoters. A widely used example is the pSV2-*neo* plasmid which has the bacterial neomycin phosphotransferase gene under control of the SV40 early promoter and confers in mammalian cells resistance to G418 (an antibiotic related to neomycin). A number of other variations may be employed to enhance expression of the selectable markers in animal cells, such as the addition of a poly(A) sequence and the addition of synthetic translation initiation sequences. Both constitutive and inducible promoters may be used.

The DNA is preferably modified by homologous recombination. The target DNA can be in any organelle of the HSC or HPC including the nucleus and mitochondria and can be an intact gene, an exon or intron, a regulatory sequence or any region between genes.

Homologous DNA is a DNA sequence that is at least 70% identical with a reference DNA sequence. An indication that two sequences are homologous is that they will hybridize with each other under stringent conditions (Sambrook *et al.*, 1990, *supra*).

The present invention further provides a pharmaceutical kit comprising reagents and/or

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compartments adapted for use in isolation of HSCs and/or HPCs from peripheral blood or bone marrow, their genetic manipulation to express DNA encoding proinsulin or an antigenic part thereof or another autoantigen associated with autoimmune diabetes and/or means to reintroduce the genetically modified cells to a subject, either to the peripheral  
5 blood system or to bone marrow.

The present invention is further described by the following non-limiting Examples.

## EXAMPLE 1

### *General methods*

#### *Mice*

- 5 NOD (non-obese diabetic) and NOD.scid mice were bred under specific-pathogen free conditions. NOD mice transgenic for mouse proinsulin II (NOD-PI) under control of the MHC class II (I-E $\alpha$ ) promoter (French *et al.*, *Diabetes* 46: 34-39, 1997) were used after breeding to homozygosity. As the incidence of spontaneous diabetes is highest in wild-type  
10 NOD females, only females were used as recipients and bone marrow (BM) donors.

#### *Antibodies and flow cytometry*

- Flow cytometric analysis was performed as described (Steptoe *et al.*, *J. Immunol.* 168:  
15 5032-5041, 2002). The following mAbs were purified from tissue culture supernatants and then used in conjugation reactions:: anti-CD3 (KT3), CD11b (5C6 or M1/70), B220 (RA3.6B2), Gr-1 (RB6-8C5), Ly-76 (TER-119), c-kit (ACK-2). Anti-CD3 (145-2C11), SCA-1 (E13-161.7) were from Pharmingen (San Diego, CA). mAb to CD4 (CT-CD4), CD8 $\alpha$  (CT-CD8a) and FITC, PE and Tricolor streptavidin conjugates were from Caltag  
20 (Burlingame, CA). For analysis of PBL mice were bled by retro-orbital venous sinus puncture with a fine glass capillary tube. Blood was collected in Alsever's anticoagulant, erythrocytes lysed and leukocytes stained and analyzed by flow cytometry. Leukocyte number determined with a hemocytometer was calibrated according to blood volume obtained. To control for inter-experimental variation, three age-matched female NOD were  
25 included in each analysis. Spleens were pressed through stainless steel mesh and cells suspended in RPMI containing 10% v/v FCS.

#### *BM preparation and transfer*

- 30 Mice (8-12 weeks old) were euthanased and femurs and tibiae collected into cold mouse-tonicity phosphate buffered saline (PBS). BM was flushed with ice cold PBS containing

2.5% v/v FCS (F2.5) (Trace Scientific, Melbourne Australia) and erythrocytes removed by  $\text{NH}_4\text{Cl}$ /TRIS buffer lysis. BM was washed in F2.5 and collected by centrifugation. For T cell depletion, BM was resuspended in F2.5, incubated with anti-CD3 mAb (KT3, 5  $\mu\text{g}/\text{ml}$ ) for 30 minutes at  $4^\circ\text{C}$ , then washed in F2.5. Antibody-labeled cells were depleted  
5 with anti-rat IgG immunomagnetic beads (Dynabeads, Dynal Biotech, Carlton South, Victoria, Australia). For sorted HSC or HPC, lineage marker-positive cells were depleted by immunomagnetic beads with a mix of FITC-conjugated lineage-specific mAb (KT3, M1/70, RA3.6B2, RB6-8C5, TER-119) at predetermined optimal concentrations. Remaining cells were labelled with anti-c-kit-phycoerythrin. For HSC isolation, lineage-  
10 depleted cells were also co-stained with anti-SCA-1-biotin, washed and stained with streptavidin-Tricolor.  $\text{Lin}^-/\text{c-kit}^+/\text{SCA-1}^+$  (HSC) or  $\text{lin}^-/\text{c-kit}^+$  (HPC) cells were collected by sterile sorting (FACSII, Becton Dickinson, San Diego, CA). Irradiated mice received a total of 950 cGy (Theratron  $^{60}\text{Co}$ , Theratronics, Kanata, ON, Canada) as two equal doses 2-3 hours apart. Cells ( $10^7$  BM or T cell-depleted BM, unless stated otherwise) were  
15 suspended in PBS and injected i.p. in 250  $\mu\text{l}$  or i.v. in 100  $\mu\text{l}$  for HSC ( $10^3$ ) and HPC ( $2.5 \times 10^4$ ), 1-3 hours after irradiation in the case of irradiated mice. Irradiated mice were maintained on neomycin-supplemented drinking water for 3 weeks post-BMT. Any mice showing signs of physical distress in the immediate post-BMT period were euthanased and excluded from analysis.

20

#### *T cell recall response*

Mice were immunized s.c. in the flank with 100  $\mu\text{g}$  ovalbumin (OVA) (Grade V, Sigma St Louis, MO) in Complete Freund's Adjuvant (Difco, Detroit, MI). Spleens collected 14  
25 days later from euthanased mice were pressed through stainless steel mesh and cells suspended in RPMI medium (GIBCO, Rockville, MA) containing 10% v/v FCS (Trace Scientific, Melbourne Australia),  $10^{-3}$  M sodium pyruvate,  $10^{-4}$  M non-essential amino acids (GIBCO),  $2 \times 10^{-3}$  M glutamine,  $5 \times 10^{-5}$  M 2-mercaptoethanol (Sigma). Splenocytes were plated in triplicate ( $2.5 \times 10^5$  cells/well, 200  $\mu\text{l}$ , 96 well flat-bottom plates) in the  
30 absence or presence of OVA (100  $\mu\text{g}/\text{ml}$ ). Cells were harvested on day 4 onto glass filter mats.  $^3\text{H}$ -thymidine (1  $\mu\text{Ci}/\text{well}$ ) was added during the final 18 hours of culture.

Incorporated radioactivity reflecting cell proliferation was measured in a scintillation counter (Topcount, Packard, Groningen, The Netherlands) and results expressed as mean stimulation index (SI)  $\pm$  standard deviation.

### 5 *Monitoring for diabetes*

Mice were urine tested for glucose weekly with Diastix test strips (Bayer, Pymble, NSW Australia). In glycosuric mice blood glucose was measured with a meter (Accu-Chek, Roche, Castle Hill, NSW, Australia). Mice were considered diabetic when two consecutive  
10 blood glucose readings were  $>12.0$  mM. Mice were euthanased when diabetic or showing sign of physical distress.

### *Assessment of insulinitis and sialitis*

15 Pancreata were removed from euthanased mice and placed in Bouin's fixative for 24 h and then transferred to 70% v/v ethanol. Fixed tissues were embedded in paraffin and H&E stained sections separated by 250-300  $\mu$ m were prepared. Insulitis was scored in a masked fashion as described (Leiter, *Proc. Natl. Acad. Sci. USA* 79: 630-634, 1982). Sublingual glands were removed and prepared as for pancreata. The number of inflammatory foci  
20 present were counted and expressed as a mean per section.

### *Statistical analysis*

Comparison of Kaplan-Meier survival curves was performed using the log-rank test  
25 (GraphPad Prism, GraphPad Software Inc., San Diego, CA). Insulitis scores between BMT groups were compared by Student-*t*-test.

## EXAMPLE 2

### *Transplantation of NOD-PI bone marrow prevents diabetes*

Whole BM from NOD or NOD-PI mice was transplanted to 4 week-old irradiated female  
 5 NOD recipients. While the onset was delayed slightly, the overall incidence of diabetes in  
 NOD BM recipients (7/12) was similar to untreated controls (15/23) (Figure 1A). In  
 contrast, diabetes was almost completely prevented in recipients of NOD-PI BM (1/16,  
 $P=0.0032$ ) (Figure 1A). NOD mice have an inherently high risk of thymoma development  
 that is exacerbated by impaired immune surveillance or exposure to ionising radiation  
 10 (Prochazka *et al.*, *Proc. Natl. Acad. Sci. USA* 89: 3290-3294, 1992; Shultz *et al.*, *J.*  
*Immunol.* 164: 2496-2507, 2000). Exclusion of mice diagnosed with thymomas at  
 necropsy increased the proportion of mice with diabetes in both groups (NOD 7/10, NOD-  
 PI 1/5) but the difference in diabetes incidence remained significant between groups  
 ( $P=0.041$ ). Because of their longer diabetes-free survival time, recipients of NOD-PI BM  
 15 had a higher proportion of thymomas (11/16) compared to NOD BM recipients (2/12).

## EXAMPLE 3

### *T cell-depletion does not modify the protective effect of transplanted NOD-PI BM*

20 Separate studies had found that mature T cells in NOD BM were capable of transferring  
 diabetes to immune-deficient NOD.scid mice. Diabetes development, following transfer of  
 whole BM, might, therefore, have reflected the diabetogenic potential of transferred  
 mature T cells. However, it was found that, whereas whole NOD BM transferred diabetes  
 to at least 50% of non-irradiated T cell-deficient NOD.scid mice, no mice that received  
 25 BM from NOD-PI mice developed diabetes.

As mature T cells in NOD, but not NOD-PI, BM could transfer diabetes, the effect of  
 transplanting T cell-depleted BM to irradiated 4 week-old mice was tested. Recipients of T  
 cell depleted NOD BM developed diabetes at a rate and incidence (10/15) similar to  
 30 untreated controls (7/12) (Figure 1B). In contrast, diabetes development was significantly  
 less (3/17,  $P=0.003$ ) in recipients of T cell-depleted NOD-PI BM (Figure 1B). When mice

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with thymomas apparent at necropsy were removed from the analysis the difference between groups remained statistically significant ( $P=0.012$ ).

Cellular immune infiltration of pancreatic islets (insulitis) was assessed 100 days post-BMT ( $1 \times 10^7$  T cell-depleted BM) In recipients of NOD-PI T cell-depleted BM, 54% of islets were free of insulitis (Figure 2A) and mononuclear cell infiltration was restricted to the islet periphery (peri-insulitis). In contrast, in recipients of NOD T cell-depleted BM only 28% of islets were free of insulitis and there was extensive infiltration into the islets (Figure 2B). These observations were reflected by a significantly reduced mean insulitis score for NOD-PI compared to NOD recipients ( $P=0.008$ ) (Figure 2C). Similar results were observed after transplantation of  $5 \times 10^6$  T cell-depleted BM. In contrast to insulitis, mononuclear cell infiltration of the sublingual gland (sialitis) was similar in recipients of either NOD-PI or NOD T cell-depleted BM and age-matched unmanipulated controls (Figure 2D). This indicates that NOD-PI BM transfer protects specifically against islet auto-immunity.

### EXAMPLE 3

#### *PI-encoding hematopoietic stem and progenitor cells transfer diabetes prevention*

Hematopoietic stem cells ( $\text{lin}^-/\text{c-kit}^+/\text{SCA-1}^+$ ) or progenitor cells (HPC) ( $\text{lin}^-/\text{c-kit}^+$ ) were sterile-purified from NOD and NOD-PI BM. To determine their effect on the development of diabetes, small numbers of either HSC or HPC were transplanted into irradiated 4 week-old recipients. Hematopoietic reconstitution was rapid and PBL populations were restored by 8 weeks post-BMT. Diabetes was totally prevented in recipients of NOD-PI HSC and its incidence significantly reduced in recipients of NOD-PI HPC (Figure 3).

### EXAMPLE 4

#### *Diabetes prevention by NOD-PI BM is not due to impaired immune reconstitution*

To exclude the possibility that the protective effect of NOD-PI BMT was the result of impaired immune reconstitution, peripheral blood leucocyte (PBL) populations were first



analysed. Ten to fourteen days post-T cell-depleted BMT, circulating leucocytes were substantially reduced in number in both NOD and NOD-PI recipients (Figure 4A). The proportions of T lymphocytes (CD4<sup>+</sup>, CD8<sup>+</sup>) and B lymphocytes (B220<sup>+</sup>) were reduced (50-75%, 25% and 80-85%, respectively) relative to age-matched controls, whereas the proportion of myeloid (CD11b<sup>+</sup>) cells was increased ~2.5-fold. At 8 and 16 weeks post-BMT, total PBL count (Figure 4A) and the relative proportion of PBL subsets (Figure 4B) were normal, indicating similar reconstitution between groups. The ability of BMT recipients to mount a T cell-mediated immune response was then investigated. Normal age-matched NOD mice and recipients of NOD or NOD-PI T cell-depleted BM were immunized with ovalbumin (OVA) 100 days post-BMT. Two weeks later, *in vitro* recall responses to OVA were similar in untreated mice and either BMT group (Figure 5). Thus, NOD-PI BMT was not associated with evidence of impaired immune reconstitution.

#### EXAMPLE 5

##### 15 *Cytokine-stimulated myeloid cells comprise undifferentiated DC precursors*

As a source of 'immature' DC (iDC), BM was cultured in GM-CSF and TGF-beta (G+T). iDC from G+T BM expressed low levels of MHC class II and co-stimulation molecules (Figure 6) and were weak stimulators in the mixed lymphocyte reaction. G+T BM from NOD-PI, but not control NOD mice, significantly inhibited ( $p < 0.01$ ) diabetes development when transferred i.v. to 4 week-old female NOD mice (Figure 7). Further investigation revealed that G+T BM contained large numbers of undifferentiated CD11c<sup>+</sup>/CD11b<sup>+</sup>/Gr-1<sup>+</sup> myeloid cells in addition to CD11c<sup>+</sup>/CD11b<sup>+</sup>/Gr-1<sup>-</sup> iDC (Figure 8).

#### 25 EXAMPLE 6

##### *Precursor DC*

Depletion of Gr-1<sup>+</sup> cells reduced the ability of G+T BM to inhibit diabetes, whereas depletion of CD11c<sup>+</sup> iDC did not (Figure 9). Transfer of purified Gr-1<sup>+</sup> myeloid cells from NOD-PI ( $p < 0.01$ ) but not control NOD mice inhibited diabetes development in recipient mice, confirming the protective role of these cells (Figure 10).

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Unlike iDC, CD11c<sup>-</sup>/CD11b<sup>+</sup>/Gr-1<sup>+</sup> myeloid cells did not rapidly acquire a mature CD11c<sup>+</sup>/CD86<sup>hi</sup> phenotype in response to activational stimuli (LPS, anti-CD40). Instead, they gradually acquired mature DC characteristics over 5-7 days in culture in GM-CSF/IL-4/TNF- $\alpha$ . CD11c<sup>-</sup>/CD11b<sup>+</sup>/Gr-1<sup>+</sup> cells present in G+T BM cultures therefore represent DC precursors. Hence, the foregoing data indicate that myeloid DC precursors encoding a disease-specific autoantigen (proinsulin) are able to prevent autoimmune disease.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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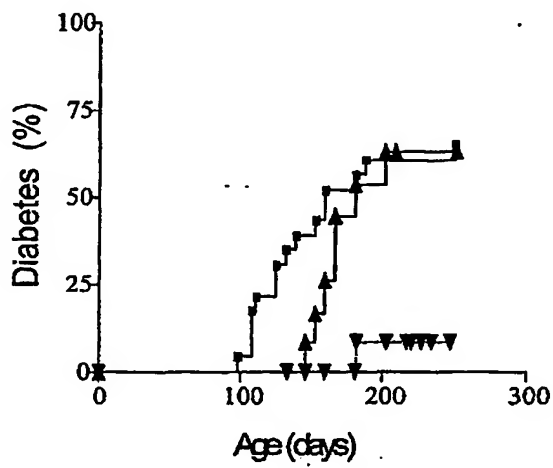
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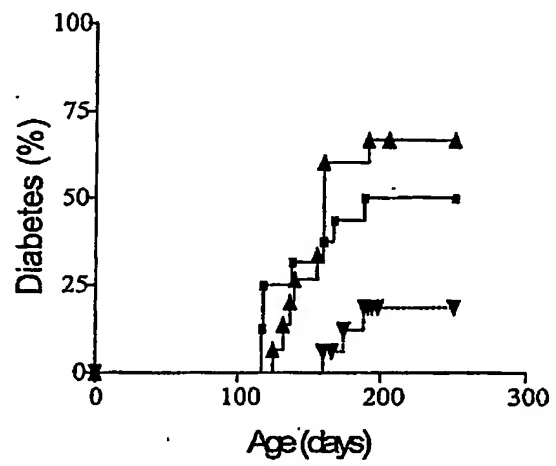
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DATED this sixteenth day of September 2002.

**The Walter and Eliza Hall Institute of Medical Research**  
by DAVIES COLLISION CAVE  
Patent Attorneys for the Applicant



**Figure 1A**



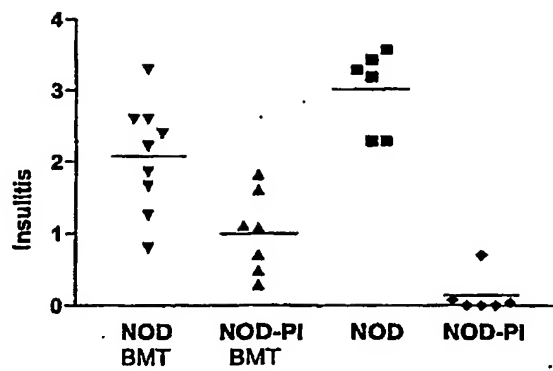
**Figure 1B**



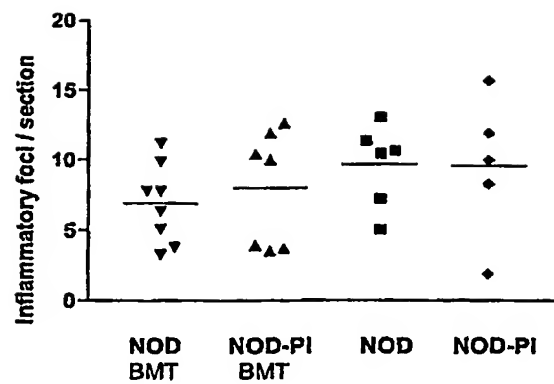
**Figure 2A**



**Figure 2B**

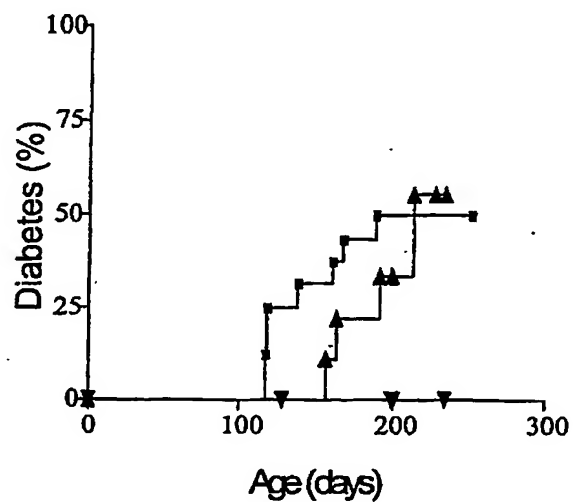


**Figure 2C**

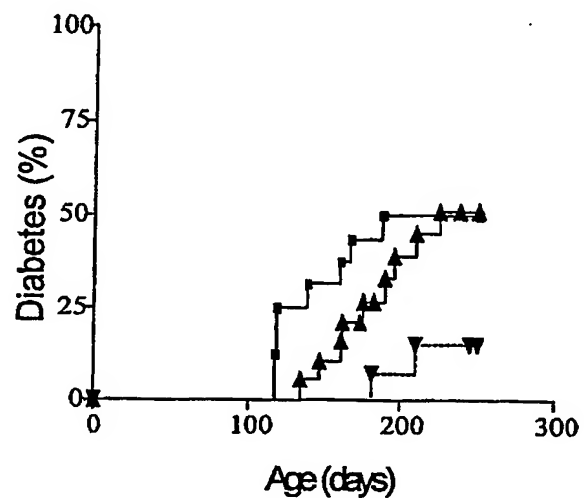


**Figure 2D**

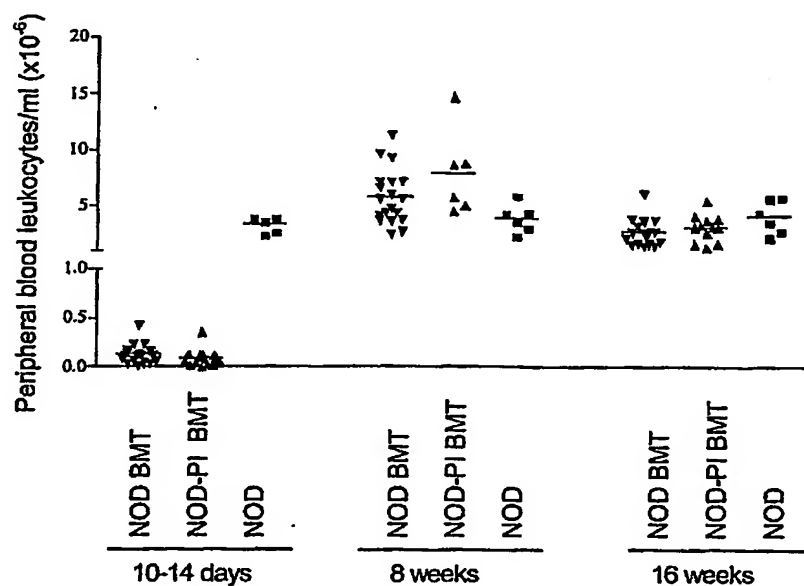




**Figure 3A**



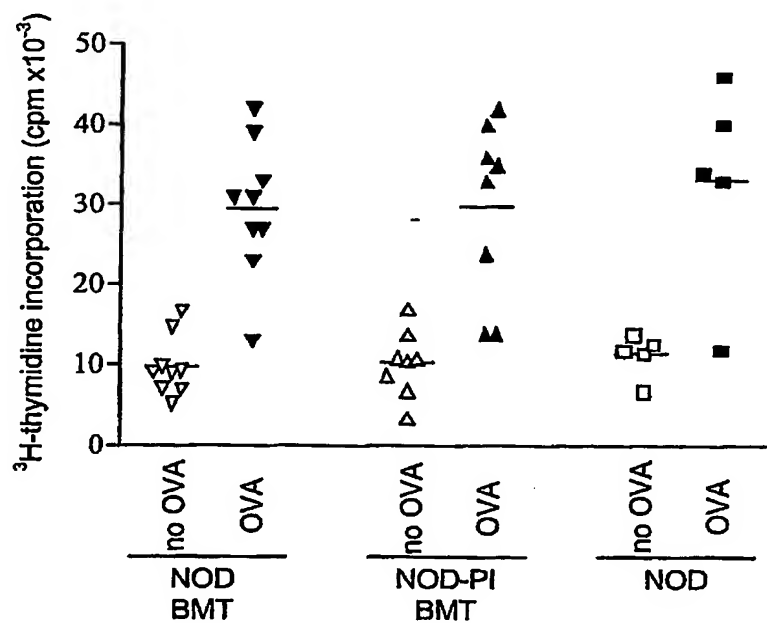
**Figure 3B**



**Figure 4A**

	8 weeks post-BMT			16 weeks post-BMT		
	NOD BMT	NOD-PI BMT	NOD	NOD BMT	NOD-PI BMT	NOD
CD3 <sup>+</sup>	38.6 $\pm$ 7.3	35.6 $\pm$ 6.0	38.7 $\pm$ 13.5	53.8 $\pm$ 13.4	43.8 $\pm$ 14.4	57.3 $\pm$ 13.5
CD4 <sup>+</sup>	29.1 $\pm$ 7.1	29.3 $\pm$ 3.1	27.5 $\pm$ 5.4	38.7 $\pm$ 12.0	32.7 $\pm$ 12.0	39.4 $\pm$ 11.8
CD8 <sup>+</sup>	8.8 $\pm$ 2.3	8.9 $\pm$ 1.3	15.8 $\pm$ 5.1	14.2 $\pm$ 3.2	11.5 $\pm$ 3.0	16.2 $\pm$ 1.2
B220 <sup>+</sup>	14.3 $\pm$ 4.8	9.1 $\pm$ 3.2	15.5 $\pm$ 2.5	16.8 $\pm$ 5.5	17.4 $\pm$ 4.4	19.7 $\pm$ 2.6
CD11b <sup>+</sup>	35.3 $\pm$ 12.1	30.6 $\pm$ 10.5	21.9 $\pm$ 11.6	22.3 $\pm$ 16.2	30.4 $\pm$ 15.3	21.8 $\pm$ 20.3
n	19	6	6	18	13	6

**Figure 4B**



**Figure 5**

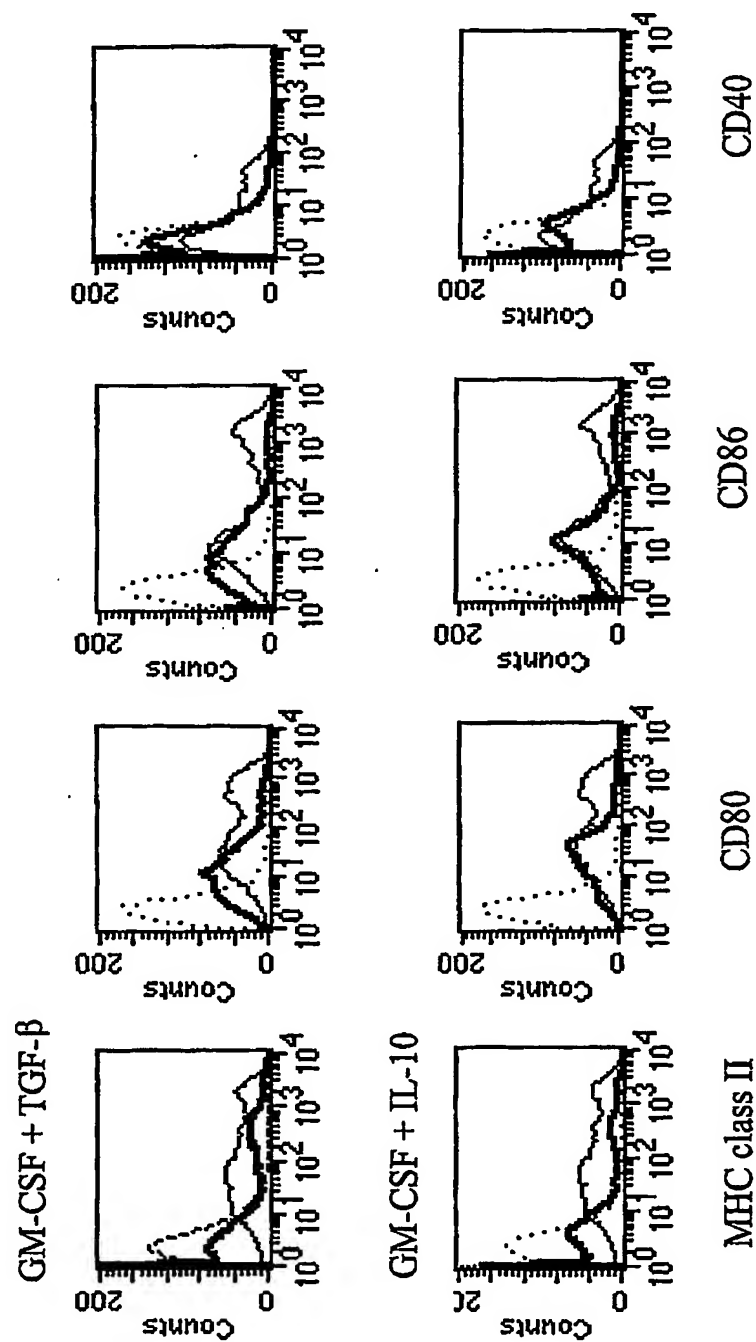


Figure 6

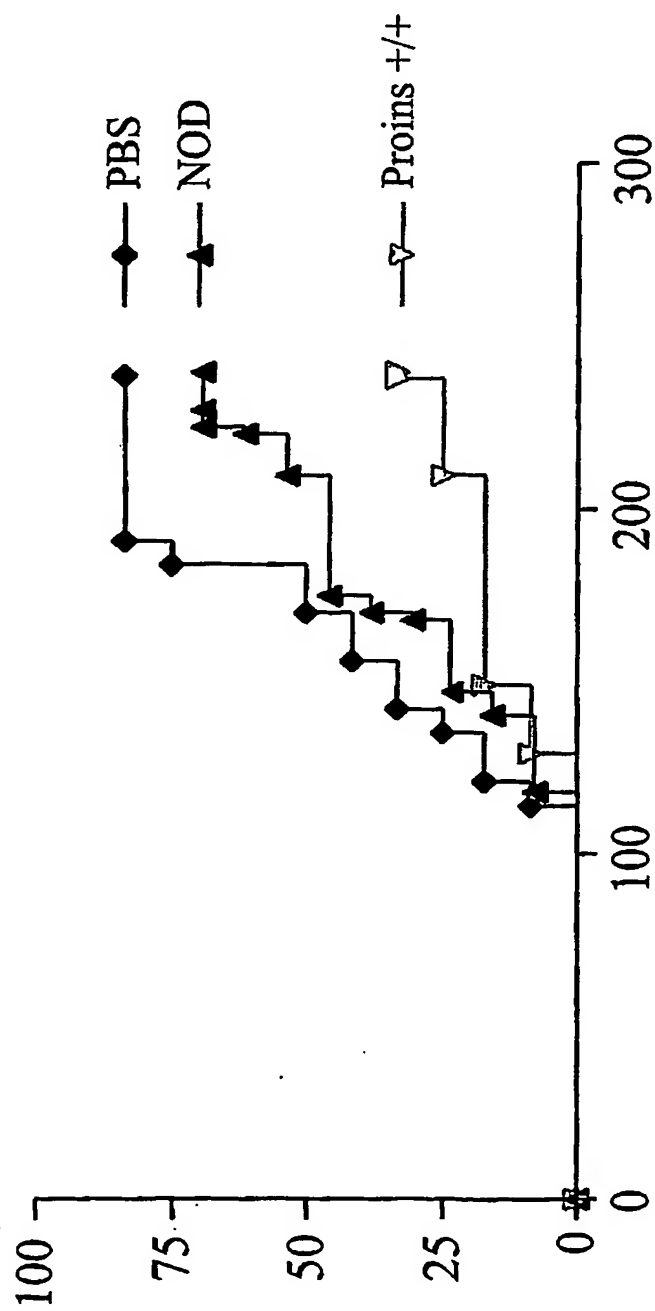
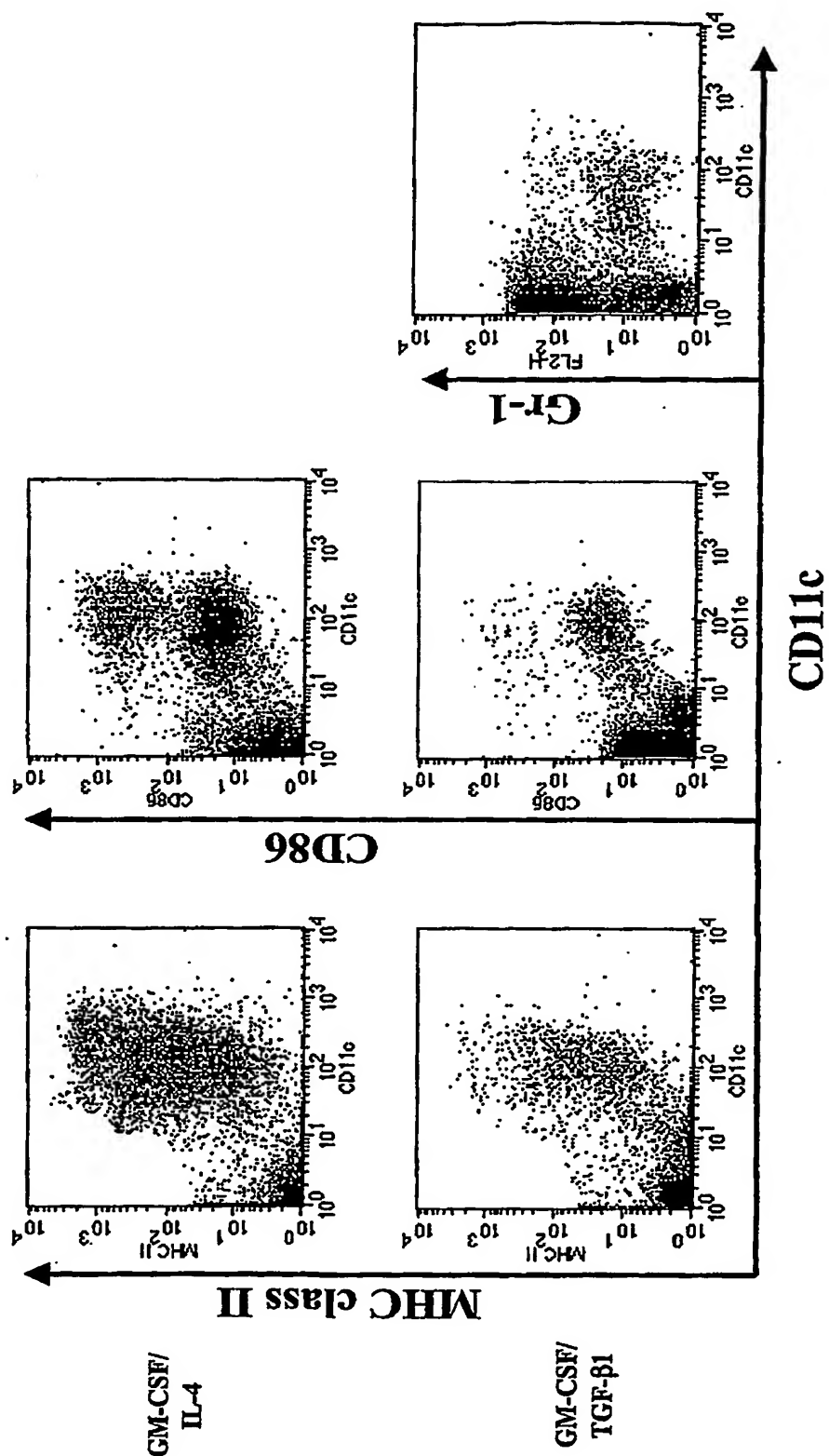


Figure 7



**Figure 8**

Treated @ 4wks

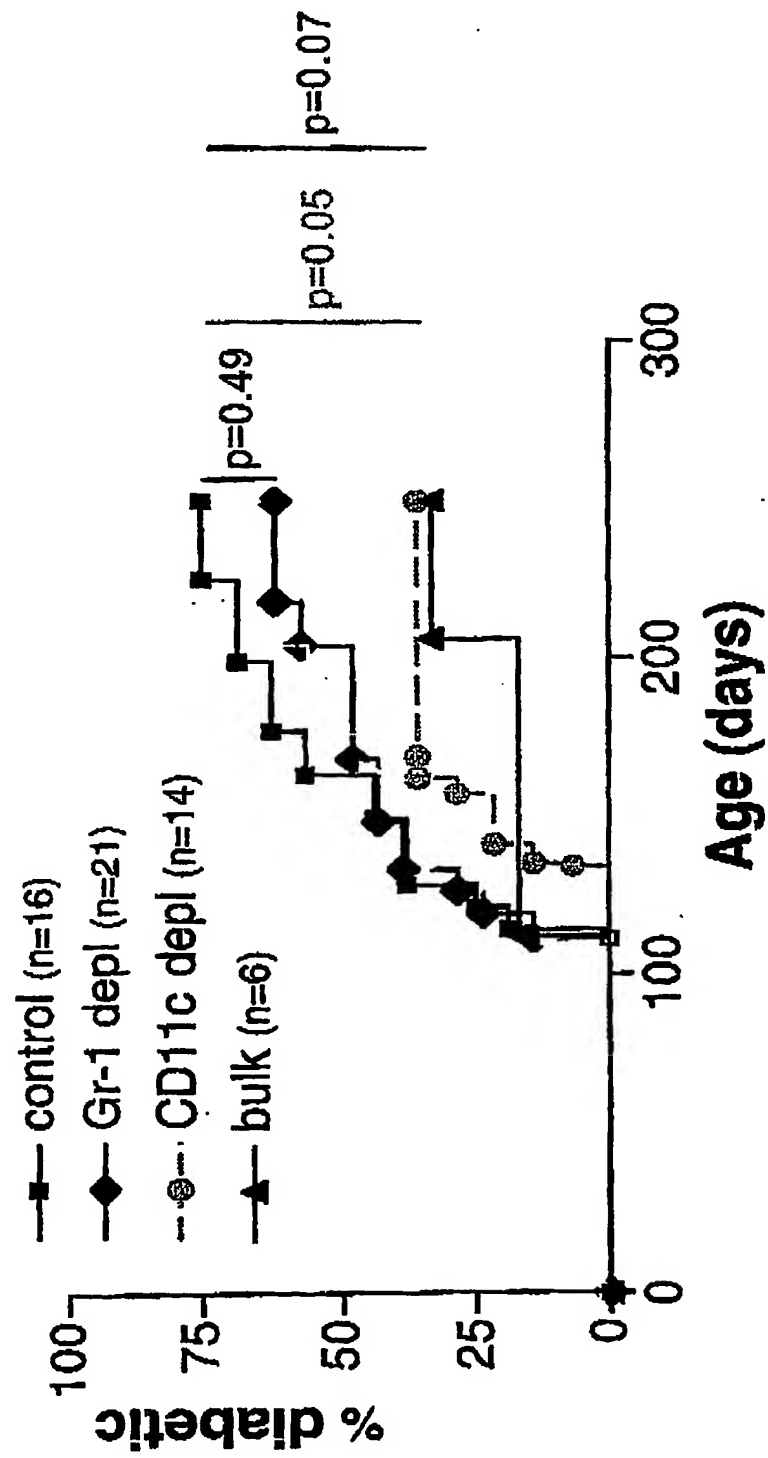


Figure 9

Treated @ 4wks (1.8 x 10<sup>6</sup> CD11c-depleted I.V.)

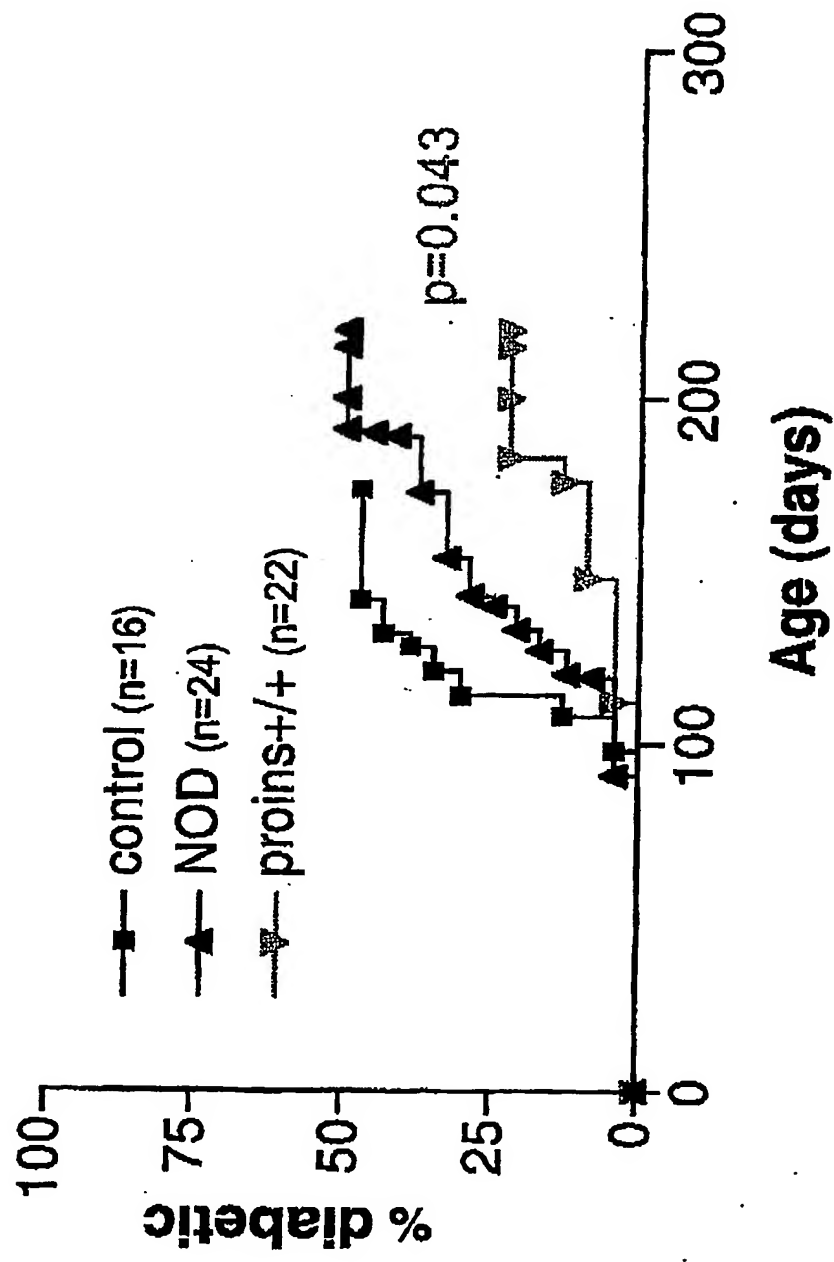


Figure 10



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